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Effects of Nasal Saline Spray on Human Neutrophils

Mark Boston, MD; Eric J. Dobratz, BS; E. Stephen Buescher, MD; David H. Darrow, MD, DDS

From the Department of Otolaryngology-Head and Neck Surgery (Drs. Boston and Darrow and Mr. Dobratz) and the Center for Pediatric Research (Dr. Buescher), Eastern Virginia Medical School and Children's Hospital of the King's Daughters, Norfolk, Virginia. Dr. Boston is currently with the Department of Otolaryngology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

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Send Correspondence to: Mark Boston, MD
Department of Otolaryngology-Head and Neck Surgery
Children's Hospital Medical Center
3333 Burnet Avenue
Cincinnati, Ohio 45229-3039
E-mail: meboston@worldnet.att.net

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ABSTRACT

Objective: Nasal saline spray (NSS) used in the treatment of rhinitis and sinusitis often contains the preservative benzalkonium chloride (BKC). Previous studies have shown that steroid nasal sprays and topical decongestants containing BKC damage respiratory mucosa, decrease mucociliary activity, and inhibit neutrophil functions in vitro. This study evaluates the effects of NSS with BKC on human neutrophils. **Design:** Prospective, basic science observations. **Methods:** Human neutrophils were exposed to NSS with BKC or phosphate buffered saline (PBS) at varying times and concentrations. The cells were examined for morphological changes by light microscopy, and for viability as determined by trypan blue exclusion. Lactate dehydrogenase (LDH) levels were measured to quantify neutrophil cell lysis. In vivo morphologic changes were studied in neutrophils obtained from the oral mucosa in human volunteers who rinsed their mouths with either NSS or PBS. **Results:** Neutrophils exposed to NSS concentrations as low as 15% showed near total cell lysis and neutrophils exposed to 20% NSS demonstrated no cell viability by trypan blue staining. PBS-exposed cells were unaffected. Release of LDH from lysed neutrophils increased sharply at NSS concentrations >10% but remained stable in PBS-exposed cells. All neutrophils isolated from NSS oral rinses were lysed, while neutrophils from PBS rinses showed an average of 78% normal morphology. **Conclusions:** NSS with BKC is toxic to human neutrophils even at concentrations far lower than those found in commercially available preparations.

Saline solutions without BKC appear to be safer alternatives and additional studies are needed to determine the clinical significance of these findings.

INTRODUCTION

All multi-use aqueous nasal sprays, including steroid nasal sprays, topical decongestants, and nasal saline solutions, contain buffering agents and preservatives. The role that these additional agents might play in either causing or exacerbating rhinitis symptoms has been debated for some time.

Nasal saline spray (NSS) is often used for relief of nasal dryness, and to debride the nose and restore ciliary function in the treatment of sinusitis and rhinitis. NSS is available as an over-the-counter preparation and is typically considered to be without harmful side effects. However, in addition to buffered saline, most NSS preparations contain the preservative benzalkonium chloride (BKC). BKC is a quaternary ammonium antimicrobial agent found in numerous medications including nasal sprays, ophthalmic drops, and bronchial inhalers. Studies of topical decongestants containing oxymetazoline chloride or xylometazoline chloride, as well as BKC or thiomersal as preservatives, have been shown to inhibit neutrophil functions in vitro.[1-3] The authors also concluded that BKC was the most toxic component in the nasal preparations studied.[3] Steroid nasal compounds preserved with BKC have also been shown to reduce mucociliary activity, damage human respiratory mucosa, and decrease neutrophil functions in vitro.[4-5] In addition, Graf et al.[6] showed that decongestant sprays containing BKC produced more rebound swelling than decongestant sprays without BKC in healthy volunteers.

Other investigators, however, have found no deleterious effects associated with BKC. Ainge et al.[7] and Braat et al.[8] demonstrated no changes in

the nasal ciliary function of monkeys or humans exposed to topical preparations containing BKC. Similarly, McMahon et al.[9] and Storaas et al.[10] failed to show any significant effects on human nasal mucosa exposed in vivo to BKC-containing nasal sprays.

Although numerous studies have examined the potential benefits of saline nasal irrigation, to date no one has specifically addressed the potential harmful side effects of NSS.[11] In this study, the morphological changes, cell viability, and lactate dehydrogenase (LDH) activity of human neutrophils exposed to either NSS or buffered saline without preservatives were examined.

MATERIALS AND METHODS

Reagents and chemicals

Dextran, Ficoll, NaCl, Triton X-100 and LDH optimized bioassay was purchased from Sigma Chemical Company (St. Louis, MO). Hanks balanced salt solution (HBSS) and phosphate buffered saline (PBS) was purchased from Bio-Whitaker (Walkersville, MD). Hypaque 76 was purchased from Sanofi Winthrop Pharmaceuticals (New York, NY) and heparin was purchased from Elfing-Sinn (Cherry Hill, NJ). Nasal saline sprays used included Deep Sea from Major Pharmaceuticals (Livonia, MI) and Ayr from B.F. Ascher & Co. Inc. (Lenexa, KS).

Purification of neutrophils

Heparinized venous blood samples were obtained from healthy adult volunteers with approval of our Institutional Review Board. The blood was

separated by Hypaque-Ficoll step-gradient centrifugation, dextran sedimentation and hypotonic lysis. The cells were then resuspended in HBSS at a concentration of 1 x 10^6 /mL. These steps provide a cell preparation that is typically >95% neutrophils by modified Wright-Giemsa stain morphology. All cells were used the same day they were obtained. Each experiment was performed with neutrophils isolated from a different donor to ensure that the observed effects were not donor specific.

Preparation of chemicals

Neutrophils were exposed to one of two commercially available NSS containing BKC, or one of two control solutions containing PBS (1X) without BKC or a "homemade" saline solution (HSS) without BKC. HSS was prepared by mixing 650 mg of NaCl with 100 ml of deionized water. All the solutions had similar pH levels (6.5), although the HSS was slightly more acidic (5.5 – 6.0). NSS, HSS, and PBS were diluted in HBSS and incubated with 50 µL of neutrophil suspension (0.5 x 10^6 cells) to obtain final dilutions of 1%-20% and a cell concentration of 5 x 10^6 /mL. The NSS used in the experiments contains BKC at a concentration of 0.01%. The final concentration of BKC in the experimental conditions is therefore .0001% to .002%. After incubation at the desired concentration and exposure time, 400 µL of HBSS was added to terminate the incubation, bringing the final concentration of cells to 1 x 10^6 /mL and the concentration of NSS, HSS, or PBS from 0.2% to 4%, during analysis.

Freshly prepared buffers were used for each experiment and all incubations were performed at room temperature (24º C) in covered cuvettes.

In vitro exposure of neutrophils and analysis

Morphological changes were studied by exposing neutrophils to NSS or PBS at concentrations of 1%, 5%, 10%, 15%, and 20% for three minutes. Five exposures at each saline concentration were performed. Cells were analyzed for morphological changes by Giemsa stain and light microscopy. Those demonstrating abnormal cell membrane borders, loss of characteristic staining pattern, or atypical nuclei were considered morphologically abnormal. Trypan blue exclusion was used to assess neutrophil viability. Equal volumes of cell suspension and trypan blue were mixed on a microscope slide and then examined by light microscopy by two independent observers. Living cells with intact cell membranes do not take up the dye, while non-viable cells are unable to exclude the dye and stain dark blue.

A quantitative determination of neutrophil cell lysis was performed using an optimized bioassay to detect lactate dehydrogenase (LDH) released from lysed cells. Neutrophils were exposed to NSS or PBS at concentrations of 1%, 5%, 10%, 15%, and 20% for three minutes. Seven exposures at each saline concentration were performed. Neutrophils were also exposed to a constant concentration (15%) of NSS or PBS for time periods of 3, 10, and 30 minutes; five exposures were performed at each time interval in this experiment. Samples

were centrifuged to remove cellular debris. Standardized aliquots of the suspension were added to the LDH kit reagents and the LDH released by disrupted neutrophils into solution was measured by spectrophotometry. Maximal LDH level was determined by exposing 0.5 x 10^6 neutrophils to 20% Triton for five minutes to lyse all the cells.

In vivo exposure of neutrophils and analysis

In vivo effects of NSS were studied in healthy volunteers who used a mucosal rinse of NSS or PBS. Oral rinses were used in order to simplify the exposure and isolation of neutrophils. Volunteers were asked to refrain from eating, drinking, smoking or brushing their teeth for one hour prior to the mouth rinse. Volunteers first rinsed with 15 mL of distilled water for five seconds to clear lose debris from their mouths. They then vigorously rinsed with 15 mL of PBS or NSS for 30 seconds and expectorated into a 50 mL tube. HBSS was added to the tube to bring the total volume to 50 mL and the tube was centrifuged at 1500 rpm for five minutes at 25°C. The supernatant was removed and the pellet was washed with 50 mL of HBSS. The tube was centrifuged again, the supernatant was removed, and the pellet was resuspended in 15 mL of HBSS. The PBS and NSS rinses were performed in the mornings on separate days.

For analysis, a 100 µL sample of the washed cell suspension was plated with a cytospin, stained, and examined by light microscopy. There were various cells in these preparations, including bacteria, mucosal epithelial cells, and

neutrophils. One hundred neutrophils were counted under high power and classified as having either normal or abnormal morphology. The counts were performed independently by two observers and the average of the two counts was recorded.

Statistical Analyses

Differences in LDH activity were examined using the Wilcoxon rank-sum test. An α value $< .05$ was considered significant. The data median and range are presented.

RESULTS

In vitro neutrophil morphology and viability

Neutrophil morphology was dramatically altered by exposure to nasal saline spray (Figure 1). Cells exposed to 20% PBS solution for three minutes exhibited normal cellular architecture with sharp, distinct cell membrane borders, characteristic staining patterns, and typical multi-lobulated nuclei (Figure 1A). Neutrophils exposed to 20% NSS for three minutes demonstrated severe disruption of cell membranes and intracellular structures (Figure 1B). Similar effects were seen at lower concentrations of NSS, although more intact cells survived.

Neutrophils exposed to 20% PBS remained viable and did not stain with trypan blue (Figure 2A), while cells exposed to 20% NSS stained dark blue (Figure 2B), indicating disruption of their membranes. Similar effects were seen

with lower concentrations of NSS but there was less extensive staining at lower concentrations.

Quantification of neutrophil cell death

Average LDH activity resulting from neutrophil lysis was graphed as a function of increasing concentration of NSS and PBS with a constant incubation time of three minutes (Figure 3). Neutrophils exposed to NSS at increasing concentrations demonstrated a significant increase in LDH activity at concentrations above 5% ($p<.05$). The cells exposed to PBS showed small amounts of LDH activity and little increase in activity with increasing concentrations of PBS.

Figure 4 shows mean neutrophil LDH activity as a function of increasing exposure time to 15% NSS and 15% PBS. Neutrophils that were exposed to 15% NSS demonstrated an increase in LDH activity with increasing incubation time. Cells exposed to 15% PBS showed minimal LDH activity and no increase over time.

In both experiments, the neutrophils exposed to 20% NSS for three minutes or 15% NSS for 30 minutes demonstrated LDH activity approaching maximal LDH levels (80-120 U/L) for the experiment as determined by Triton cell lysis. Experiments performed with HSS showed levels of LDH activity similar to PBS (data not shown). The data presented are from one brand of NSS only. The results from the second brand are similar but are not presented.

In vivo neutrophil morphology

Neutrophil morphology was significantly altered in the cells isolated from the NSS oral rinses as compared to the cells isolated from the PBS oral rinses. Cell counts from the two oral mucosal rinses are shown in Figure 5. Neutrophils isolated from the NSS rinse exhibited 100% abnormal morphology, while an average of only 22% of those isolated from the PBS rinse were considered abnormal cells.

COMMENT

Nasal saline spray is available without a prescription and is frequently used in the medical treatment of sinonasal disease and following sinus or nasal surgery. Potential benefits of NSS include moistening of dry nasal mucosa, clearance of debris from the nasal passageways, and improved mucociliary function. NSS is conveniently packaged in multi-use bottles that make administration simple and relatively inexpensive. However, multi-use containers are subject to bacterial growth over time unless antimicrobial preservatives are used in the solutions. The most frequently used preservative in NSS and other aqueous nasal sprays is BKC. Previous studies have documented the cytotoxic effects of BKC on neutrophils and nasal mucosa.[1-5]

The present study demonstrates that NSS containing BKC, even at concentrations far lower than those in commercially available preparations, alters neutrophil morphology, decreases cell viability and increases neutrophil LDH activity in a concentration and time dependent manner. We also found that NSS

is toxic to neutrophils in an *in vivo* model by exposure to oral mucosa. None of these effects were seen with PBS, which is similar in composition to NSS but without the preservative, BKC. In vitro experiments were repeated with an unbuffered "homemade" saline solution, not containing preservative, which produced results similar to PBS.

Neutrophils are the primary cellular mediators of acute inflammation and are found abundantly on the mucosal surfaces of the nose and oral cavity. These cells provide a front line of defense against invading pathogens through their functions of phagocytosis and degranulation. Functional neutrophils must respond in adequate numbers if the body is to successfully combat infectious processes including sinusitis. BKC appears to inhibit this process. Furthermore, the release of inflammatory mediators from lysed neutrophils into surrounding tissues may result in increased vascular permeability and recruitment of other neutrophils and immune cells to the area. Therefore, it is possible that the lysis of mucosal surface neutrophils can result in a local inflammatory response in the absence of pathogenic microorganisms. Because NSS is often used four or more times a day, the clinical significance of neutrophil cell lysis may be greater than for nasal sprays used only once or twice a day.

Eosinophils also play a crucial role in the pathogenesis of rhinitis, especially in the allergic patient. Neutrophils, however, outnumber eosinophils several-fold and are abundantly found on the mucosal surfaces lining the mouth, nose and paranasal sinuses. The lysis of neutrophils would, therefore, be expected to result in the release of a greater amount of inflammatory mediators

and possibly result in a clinical effect. Our observations of the few eosinophils and basophils that remained after neutrophil purification showed that NSS severely disrupted their cellular structure as well.

Initially we attempted to study neutrophil function by examining stimulated calcium ion release and aggregation of neutrophils following exposure to NSS. It quickly became evident, however, that the NSS exposed cells were already lysed and the data from these initial experiments were uninterpretable. It is not known if the few intact remaining neutrophils following NSS exposure continue to function normally as we are unable to isolate these cells in the lab.

This study does not specifically address the clinical significance of long-term NSS use and we are unaware of any other study that has examined the possible link between neutrophil lysis and clinical symptoms in the nose. Most patients derive significant benefit from the use of NSS despite the presence of BKC. One possible reason for this disparity may be that the mechanical washing effect of the saline spray removes the lysed cells along with other debris. Another possible explanation is that mucosal proteins in the nose may bind to BKC and inactivate it. It can be argued that evaluating the effects of NSS on neutrophils from oral rinses is not the same as examining nasal mucosa neutrophils. We concede this point and we utilized oral rinses in this study because this technique provides an abundance of neutrophils that make qualitative evaluation in our study design easier. This is the first study of which we are aware, however, that examines the effects of BKC on human neutrophils in vivo and that specifically evaluates the potential harmful side-effects of NSS.

The widespread use of NSS and common assumption that NSS is harmless necessitates a closer evaluation of its use and potential side effects. While there are few preservative-free steroid or topical decongestant preparations available, saline for nasal irrigation can easily be made at home without potentially harmful additives. Patients should be counseled regarding the need to periodically make fresh saline preparations and methods to avoid contamination. Previous studies have called for the elimination of BKC from medicinal preparations and the need for alternative preservatives has been stated.[5] Recently, Cho et al. [12] showed that steroid preparations containing either BKC or potassium sorbate, a common food preservative, caused inflammatory changes in rat nasal mucosa, although the potassium sorbate induced changes were not as severe and improved with continued exposures. Interestingly, we have found that steroid nasal spray preserved with potassium sorbate did not cause nearly as much neutrophil disruption as did steroid sprays containing BKC (unpublished observations).

CONCLUSION

We conclude that multi-use preparations of NSS are toxic to human neutrophils, and that substances used to preserve NSS, particularly BKC, are responsible for the toxicity. We believe that the lysis of neutrophils in the nasal mucosa may impair the cellular response to pathogenic organisms and lead to the release of inflammatory mediators, thus exacerbating the symptoms of rhinitis that NSS is intended to relieve. Homemade saline solutions and solutions

without BKC appear to be safer alternatives. Alternative preservatives and novel methods of preservative-free packaging of aqueous nasal sprays should be sought. Finally, further studies are needed to assess the clinical significance of these laboratory findings.

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FIGURE LEGENDS

Figure 1. Giemsa stain of neutrophils exposed to 20% PBS (A) or 20% NSS (BKC concentration .002%)(B). The PBS exposed cells appear normal, while the cells exposed to NSS show severe alteration of their cell membranes and intracellular structures. Original magnifications 1000X.

Figure 2. Trypan blue stain of neutrophils exposed to 20% PBS (A) or 20% NSS (B). The PBS exposed neutrophils have intact cell membranes and the dye is not able to penetrate the cells. The cells exposed to NSS stain dark with the trypan blue, indicating disruption of the cell membranes. Original magnifications 40X.

Figure 3. LDH concentration in solution from neutrophils exposed to varying concentrations of one brand of NSS (n=7) or PBS (n=5) for 3 minutes. Exposure to concentrations of NSS ≥ 5% (BKC concentration .0005%) caused a statistically significant increase in LDH concentration over exposure to PBS. Maximal LDH concentration was 80 – 120 U/L as measured by neutrophils lysed in Triton. Data are expressed as median and range. Significant differences indicated by * (95% confidence interval).

Figure 4. LDH concentration in solution from neutrophils exposed to either 15% NSS (n=5) or 15% PBS (n=3) over time. LDH values were measured at 3, 5, and 30 minutes after exposure. NSS exposed neutrophils showed a gradual increase in LDH activity over time whereas PBS exposed cells did not. Data are expressed as median and range. Significant differences indicated by * (95% confidence interval).

Figure 5. Bar graph illustrates mean number of abnormal neutrophils per 100 neutrophils counted from oral rinses with NSS and PBS. Nearly all neutrophils from the NSS rinses were abnormal in morphology, whereas a mean of 22% of neutrophils from the PBS rinses were abnormal. Data shown for each individual and for the mean.